CHAPTER-VI

6. DISCUSSION

Findings of the study

The present survey of traditionally used hepatoprotective medicinal plants, a sum of 40 plants from 26 different families and 40 genus were identified and documented. From the result it was revealed that the tribe uses 29 types of leaves, 10 types of roots, 3 types of whole shoot, 2 types of flowers, one type of bark, fruit and seed respectively. The plants of the family Acanthaceae is found to be the highest (with 4 plants) in use followed by Moraceae, Rubiaceae, Rutaceae with 3 plants among them. The process of medicine preparation/formulation depend on traditional healers, who may use single or combination of different plants or plant parts for treating hepatic related disorders. Out of 40 different medicinal plants collected, various literature review on medicinal plants have revealed that the plants *viz*: *Pogostemon plectranoides* Desf., *Amphineuron opulentum* (Kaulf.), *Hygrophila plomoides* Nees. and *Morinda angustifolia* Roxb., were not reported earlier as hepatoprotective medicinal plants as well as no scientific research were carried out.

In a different survey, Kpodar *et al.* 2016, Sharma *et al.* 2012 and Thokchom *et al.* 2016, reported 99 plant species (49 families & 88 genera), 40 plants (31 families & 38 genera) & 40 plants (under 22 families) are used as hepatoprotective medicinal plants by the traditional healers of Togo, Africa; Bhoxa, Tharu & nomadic Gujjars community of Sub-Himalayan region of Uttarakhand; and Meitei community of Imphal east & west districts of Manipur, India respectively. Kpodar *et al.* 2016, reported that *Caesalpiniaceae* families were the highest with 8 species, followed by *Euphorbiaceae* with 7 species and leaves followed by root parts were mainly used. As per literature supported by Sharma *et al.* 2012, the most commonly used hepatoprotective plant species in India is *Boerhavia diffusa* followed by *Tinospora cordifolia*, *Saccharum officinarum*, *Phyllanthus amarus*, *Ricinus communis*, *Andrographis paniculata*, *Oroxylum indicum*, *Lawsonia inermis* and *Eclipta prostrata*. Thokchom and co-worker 2016, found out that *Andrographis paniculata* (Burm.f.) Nees was having highest DCI (Disease concensus index) value among studied plants and also revealed that the whole plant (21%) followed by leaves & fruit (16%) were found to be highest in use.

In Bangladesh, Bin Nyeem *et al.* 2017, reported some plants to have hepatoprotective properties such as *Solanum nigrum, Berberis aristata, Rosa damascena, Tinospora cordifolia, Phyllanthus niruri, Foeniculum vulgare, Rubia cordifolia, Mimosa pudica,*

Phyllanthus emblica, Phyllanthus emblica, Nyctanthes arbortritis, Ficus hispida, Aegle marmelos, Capparis spinosa, Cassia fistula, Azadirachta indica, Andrographis paniculata etc., and *Cichorium intybus*, *Herpetospermum caudigerum* in different study by Khalid *et al*. 2018 and Cao *et al*. 2017. These medicinal plants have promising phytochemicals that have already been tested in hepatotoxicity models using modern scientific system.

Though antioxidant properties of different parts (leaves, flower, fruit and bark) of *Morus indica, Averrhoa carambola* and *Phlogacanthus thyrsiflorus* plants were reported earlier, very limited or no scientific study was done on the roots of these plants. Although a study on anti-inflammatory activity of *Morus indica* root was conducted and the results obtained from the study did showed anti-inflammatory activity of the plant.

Root extracts of *M. indica*, *P. thyrsiflorus* and *A. carambola*, showed important phyto-constituents such as phenolics, flavonoids and tannins which are major group of compounds that act as natural antioxidants or free radical scavengers (Jayshree *et al.* 2016; Song *et al*. 2010) which possess antimicrobial, anti-allergic, anti-mutagenic, anti-inflammatory and anti-carcinogenic properties (Yao *et al.* 2004; Najafabad & Jamei 2014; Anupa *et al*. 2016; Ghasemzadeh & Ghasemzadeh 2011) and also showed positive for resins, terpenoids, glycosides and steroids. In another study by Niratker & Singh 2014, phytochemical screening of leaves of *M. indica* L., have shown that methanolic and ethanolic extracts are rich in carbohydrates, saponins, alkaloids, flavonoids, proteins and amino acids, terpenoids, reducing compounds, tannins, phenols and cardiac glycosides. Presence of tannins, flavonoids, saponins, carbohydrates, steriods, alkaloids, reducing sugar, and terpenoids were reported in the methanolic leave extracts of *P. thyrsiflorus* (Das *et al.* 2015). *A. carambola* preliminary phytochemical analysis of fruit extract showed the presence of flavonoids, tannins, saponins and alkaloids (Thomas *et al.* 2008). Whereas the acetone leave extract of *A. carambola* revealed the presence of various phytoconstituents including alkaloids, tannins, reducing sugar and flavonoids in them (Mazumder & Choudhury 2013).

The compounds such as phenolic are rich in plants/plant parts (Maheshwari *et al.* 2011 & Hseih *et al.* 2016). The current study on total phenolic content were in order of RoAc-EE (235.26 ± 11.91) > RoMi-EE (214.71 ± 2.21) > RoAc-AE (213.91 ± 11.18) > RoMi-AE (190.61 ± 2.88) > RoPt-EE (101.26 ± 2.52) > RoPt-AE (84.21 ± 4.82). Phenolics such as phenolic acid, tannins, flavonoids, tocopherols etc., are natural antioxidants that possess antimicrobial, anti-allergic, anti-mutagenic, anti-inflammatory and anti-carcinogenic

properties (Yao *et al.* 2004; Najafabad & Jamei 2014). Higher amount of phenolic and flavonoid content corresponds to their stronger antioxidant capacity. Therefore, phenolics and flavonoids have many essential roles in decreasing the risk of various human diseases (Anderson *et al.* 2001; Alam *et al.* 2013; Florence 1995). Moresco *et al.* 2012, found total phenolic content of 79.07+0.63 mg/g of dry fraction from *A. carambola* leaves ethanolic extract. Antioxidant study on 56 selected Chinese medicinal plants by Song *et al.* 2010, have found highest phenolic content in the plant of *Dioscorea bulbifera* L. with 59.43 ± 1.03 and only 5.34 ± 0.09 mg GAE/g of dried extract from *Morus alba* L. (bark of root). Chanu *et al.* 2012, found total phenolics in the aqueous seed extract of *Parkia javanica* having 51.09 ± 0.78 and 48.75 ± 1.43 mg GAE/g in the *P. thyrsiflorus* methanolic leave extract. Several studies have shown that polyphenols are directly attached with several biological activities such as anti-inflammatory, hepatoprotective activity etc. (Santillan *et al.* 2014; Wu *et al.* 2017).

In the present study higher amount of total flavonoid contents are in order of RoMi-EE (123.39 ± 2.04) > RoMi-AE (113.09 ± 7.25) > RoAc-EE (101.96 ± 6.87) > RoPt-EE (99.92 ± 5.49) > RoAc-AE (82.81 ± 5.94) > RoPt-AE (68.22 ± 4.82). Hsieh *et al.* 2016 has found the maximum yield of total flavonoid in *Ajuga nipponensis* with 7.87 ± 0.10 mg/g was obtained in 70% ethanol extract when the extraction time was 50 minutes and the extraction temperature was at 60ºC. In another study by Raman *et al.* 2016, have found flavonoid content of 187.23 mg/g from the 70% ethanol flavonoid extracted from that of mulberry fruit (*Morus alba* L.). From the current study, it was found that total flavonoids in RoMi-EE is 16 folds higher than that of *A. nipponensis* ethanolic extract but was only 0.6 folds lower than the *M. alba* fruit ethanolic extract.

The reducing power activity increases with increased concentration of the extract which was comparable to that of standard. Reducing power assay acts by reducing ions or by donating electron and the antioxidants present in the plant extracts causes the reduction of Fe3+/ ferricyanide complex to the ferrous form (Ahmed *et al.* 2015; Najafabad & Jamei 2014). The activity of extracts might be due to the occurrence of flavones hydroxyl, phenolic hydroxyl or methoxyl groups, free carboxylic groups, keto groups and others such as triterpenes and their derivative (Najafabad & Jamei 2014). In the current study, the increased absorbance value of reducing power activity of the extracts were significantly lower than the standard in order of BHA (2.928 at 160 µg/mL) > RoAc-EE (0.907 ± 0.015) > RoMi-EE (0.878 ± 0.035) > RoAc-AE (0.732 ± 0.014) > RoMi-AE (0.498 ± 0.03) > RoPt-EE (0.421 ±

0.013) > RoPt-AE (0.395 ± 0.015) at 200 µg/mL concentration of dried extract. The reducing power ability of extract was compared with standard BHA at a concentration of 10-160 µg/mL at 0.278- 2.928 OD respectively. Study on *Ajuga nipponensis* reducing power activity by Hsieh *et al.* 2016, indicated that when the standard (BHA) as well as extract concentration was 5 mg/mL, the absorbance values of reducing power were (3.00 ± 0.09) mg/mL and (2.43 ± 0.04) mg/mL of BHA/ extract respectively.

Total antioxidant assay follows the principal that chemistry of conversion of Mo (IV) to Mo (V) compounds in presence of reducing agents (antioxidants) which results in formation of green phosphate/Mo (V) complex which can provide maximum absorbance at 765 nm (Ahmed *et al*. 2015; Pisoschi *et al*. 2016). In present study, highest concentration of ascorbic acid equivalent total antioxidant capacity were found to be in order of RoMi-EE (584.98 ± 22.28) > RoAc-EE (512.87 ± 29.72) > RoAc-AE (478.57 ± 24.99) > RoMi-AE (287.3 ± 17.3) > RoPt-EE (198.35 ± 18.25) > RoPt-AE (189.94 ± 16.72) mg AAE/g of the dried extracts. The data obtained are presented in the **Figure 8**. Shah *et al.* 2013, have found the highest concentration in *Sida cordata* ethanolic extract (antioxidant value of 1.129 ± 0.01) with 200 µg/mL. Sasikumar and Kalaisezhiyen 2014, have studied the total antioxidant activity of leaves of *Kedrostis foetidissima* was found to be higher in methanolic extract with (60.88 ± 1) than other extracts and lowest activity was found in the petroleum ether extract with 17.99 ± 0.66 mg of AAE/g of extract. The total antioxidant activity of the five extracts, was found to decrease in decreasing order of *K. foetidissima* Methanolic extract > *K. foetidissima* Chloroform extract > *K. foetidissima* Aqueous extract > *K. foetidissima* acetone extract > *K. foetidissima* petroleum ether extract.

DPPH possesses proton free radicals with property of absorption that decreases on exposure of proton radical scavengers (Shah *et al.* 2013; Sravanthi & Rao 2015). The results of DPPH radical scavenging activities were expressed in IC_{50} value. The IC_{50} is the inhibitory concentration of the sample having the potential to scavenge 50% reactive oxygen species (ROS) or inhibit oxidation process by 50%. IC_{50} values are inversely related to the scavenging activity, higher the IC_{50} value means lower antioxidant activity and lower the value means higher antioxidant activity. In the current study, extracts showed high DPPH radical scavenging activity with increased concentration of extracts and ascorbic acid. The ethanolic root extract of *M. indica* showed highest percent inhibition of 54.36 ± 2.15% and acetone extract of *P. thyrsiflorus* roots showed lowest inhibition with only 30.05 ± 3.56% at only 160 µg/mL concentration. The IC_{50} values were found to be in order of Ascorbic acid

(48.93 µg/mL) > RoMi-EE (130.57 ± 12.46) > RoAc-EE (138.66 ± 11.41) > RoAc-AE (174.1 ± 21.18) > RoMi-AE (233.92 ± 14.46) > RoPt-EE (265.87 ± 17.58) > RoPt-AE (302.55 ± 35.68). The radical scavenging activity of *M. indica* roots ethanolic extract was better than that of *Lippia javanica* methanol leave extract that showed best IC_{50} value of 135.00 ± 1.49 µg/mL among other plant extracts (Narzary *et al.* 2016) and was also 3.5 folds better than the water extract of *Reinwardtia indica* leaves having IC_{50} value of 450 ± 0.07 µg/mL (Shukla *et al.* 2016) and that of seed extracts of *Parkia javanica* and leave extracts of *Phlogacanthus thyrsiflorus* reported by Chanu *et al.* 2012.

The method of ABTS radical scavenging activity depends on inhibition of the absorbance of ABTS radical cation. Decolonization of ABTS reflects the capacity of the antioxidant species to donate electrons or hydrogen atoms to inactivate these free radical chain reaction or oxidation of other molecules (Pellegrini *et al.* 2003). In the presence of antioxidant molecule, the coloured radicals were converted back to colourless ABTS. The assay is widely used to evaluate the antioxidant properties of plant extracts. In the present study, RoAc-EE showed lowest IC_{50} values having percent inhibition of 97.2 ± 2.28% and RoPt-AE showed highest IC_{50} values among the extracts having inhibition of only 58.97 ± 2.19% at 32 µg/mL concentration. The IC_{50} values of the standard is comparable to that of extracts and are in order of BHT (7.04) > RoAc-EE (7.94 ± 1.33) > RoAc-AE (8.81 ± 1.66) > RoMi-EE (8.82 ± 1.42) > RoMi-AE (12.75 ± 1.61) > RoPt-EE (17.89 ± 1.18) > RoPt-AE (24 ± 1.61). Shah *et al.* 2013, reported the IC_{50} value of 143 ± 0.8 µg/mL in the ethyl acetate fraction of *Sida cordata* whole plant methanolic extract. In another study by Ismail *et al.* 2015, *Nepenthes bicalcarata* methanolic leaves extract showed IC_{50} value of 16.13 ± 0.33 µg/mL. The values of RoAc-EE, RoAc-AE and RoMi-EE were 17 folds lower than the *S. cordata* and 2 folds better than that of *N. bicalcarata*. The results were also better than ethanolic leaf extract of *Celtis toka* (48.6 ± 6.8) Fall *et al.* 2017, rhizome methanol (137.3) and ethanol extract (125.01) of *Helicoria rostrata* (Moonmun *et al.* 2017) and the ethanol fruits extract of *Artemisia nilagirica* which showed the IC_{50} value of 300 µg/mL reported earlier by Suseela *et al.* 2010.

For the oxygen transport, respiration and for many enzyme activity, the Iron (II) is very much important. Chelating agents inhibits the process of lipid peroxidation by stabilizing the transition metals (Sasikumar & Kalaisezhiyen 2014; Pavithra & Vadivukkarasi 2015; Amaral *et al*. 2018). It is reported that metal chelating ability of leaf extracts of *K.*

foetidissima, the methanolic extract was most effective having EC_{50} value 1000 µg/mL and least effective was found in petroleum ether extract with EC_{50} value 7600 µg/mL (Sasikumar & Kalaisezhiyen 2014). This might be due to increase in secondary metabolite of the extracts. The chelating agents act as secondary metabolites which reduces the redox potential by stabilizing the oxidized form of metal ion (Shukla *et al.* 2016; Pavithra & Vadivukkarasi 2015). Study from the *Morus alba* fruits flavonoid extract, Raman *et al.* 2016, found little Fe^{2+} chelating capacity at low concentration but at 6000 µg/mL FEM reached 72.6% chelating activity. The results of the present study showed concentration dependent activity. At 1000 µg/mL concentration the RoPt-EE showed 72.06 ± 6.69% highest chelating activity followed by RoMi-EE with 50.06 ± 6.08% which is far better than the results obtained by Raman *et al.* 2016. The EC_{50} value of the samples/standard were in order of EDTA (63.33) > RoPt-EE (535.16 ± 121.56) > RoMi-EE (1038.6 ± 143.97) > RoPt-AE (1471.32 ± 91.7) > RoAc-EE (1500.43 ± 130.1) > RoAc-AE (1817.3 ± 183.26) > RoMi-AE (2006.9 ± 170.4). The EC_{50} value of RoPt-EE was showing 2 times better result than the *K. foetidissima*, whereas the RoMi-EE was comparable to that of *K. foetidissima* but were higher than the *Hyoscyamus squarrosus* fruits extract reported earlier by Ebrahimzadeh *et al.* 2009.

The conversion of H_2O_2 to hydroxyl radical might be toxic to the cells which is extremely reactive free radical formed naturally in the biological system and known to implicate highly destructive among the free radical species, and therefore, its inhibition is very much important in order to protect the body cells/tissues (Oyedemi & Afolayan 2011; Ozcan & Ogun 2015; Nimse & Pal 2015). The result indicated concentration dependent activity with highest percent inhibition observed in RoAc-EE with 44.8 ± 2.93 followed by RoMi-EE of 37.85 ± 6.23 which was depicted IC_{50} value of 12.67 ± 1.58 and 12.88 ± 1.54 µg/mL with slightly lower activity than BHA having inhibition of 57.23% and IC_{50} value of 7.59 µg/mL. The results were convincingly better than *B. lanceolaria* methanolic leave extract (Narzary *et al.* 2016) having highest percent inhibition of 73.52±0.04 % with IC_{50} value of 20.37 ± 0.01 µg/mL and was also better than that of Triphala reported earlier using the same methodology by Babu *et al.* 2013, having IC_{50} value of 16.63 ± 2.01 µg/mL. Higher scavenging activity of the extracts may be attributed to phenols and tannins presence which can donate electrons and thereby converting it into water Babu *et al.* 2013. Oyedemi and Afolayan 2011, found out *Schotia latifolia* hydroalcoholic stem bark extract could scavenge the 4mM H_2O_2 radicals with inhibition up to 86.48 % at 500µg/mL concentration which was depicted to IC_{50} value of 66 µg/mL. The IC_{50} value of the samples/standard were in order of

BHA (7.59) > RoAc-EE (12.67 ± 1.58) > RoMi-EE (12.88 ± 1.54) > RoAc-AE (15.82 ± 2.13) > RoMi-AE (16.9 ± 1.8) > RoPt-EE (17.89 ± 1.05) > RoPt-AE (18 ± 1.87).

Originally, Benzie and Strain developed the FRAP assay which measures the reducing power of the plasma (Benzie & Strain 1996). The method is based on the capacity of antioxidants that has the potentials to reduce the ferric complex (Fe^{3+}/ TPTZ) to the coloured ferrous complex (Fe^{2+} /TPTZ) at pH 3.6 (Apak *et al*. 2016; Adebiyi *et al*. 2017). The result interpretation is based on the assumption that the capability of extract/antioxidants which reduces ferric ions has the ability to reduce reactive oxygen species (ROS) (Pinchuk *et al.* 2012). *Dioscorea bulbifera* showed highest antioxidant capacity of 856.92 µmol Fe^{2+}/g, followed by *Tussilago farfara* with 455.64 µmol Fe^{2+}/g and least antioxidant property in the plant *Sargassum fusiforme* with 0.15 µmol Fe^{2+}/g (Song *et al.* 2010). According to the present result, highest antioxidant activity was in order of RoAc-AE> RoAc-EE> RoMi-EE> RoMi-AE> RoPt-AE> RoPt-EE having better ferrous ion concentration with 2512.7 ± 157.37, 2484.27 ± 135.3, 1116.4 ± 98.56, 1027.9 ± 115.03, 820 ± 110.63 and 751.67 ± 85.48 µmol Fe^{2+}/g respectively and are far better than the *D. bulbifera, T. farfara, Eriobotrya japonica* (437.40 µmol Fe^{2+}/g), *Ephedra sinica* (388.68 µmol Fe^{2+}/g) and *Arctium lappa* (223.68 µmol Fe^{2+}/g) and *S. fusiforme* except RoPt-AE and RoPt-EE which is slightly lower than the *D. bulbifera.* In an antioxidant study of methanolic extracts of 50 different medicinal plants by Gan *et al.* 2010, the highest Fe (II) concentration was found in methanolic extracts of *Loranthus parasiticus* (580.02 ± 31.32) followed by *Geranium wilfordii* (347.33 ± 7.99) and the least concentration was observed in *Poria cocos* (3.88 ± 0.15) µmol Fe(II)/g and were lower than the present results. The current results also showed better Fe(II) concentration than the ethanolic extract of Brazilian native fruits reported earlier by Denardin *et al.* 2015.

GC-MS profile indicated the presence of 1,2-Bis(Trimethylsilyl) Benzene; Silane, 1,4-Phenylenebis (Trimethyl-; Alpha.-Amyrin; 2,4,6-Cycloheptatriene-1-One, 3,5-Bis-Trimethylsilyl; Octadecanoic Acid, Ethyl Ester ; N-Hexadecanoic Acid; Eicosanoic Acid; 9,12-Octadecadienoic Acid (Z,Z)-; 1-Octadecyne; Pentadecanoic Acid-; Oleic Acid-; Urs-12-En-28-Ol -25.918-; Ethyl Iso-Allocholate-; 7-Dehydrocholesteryl Isocaproate-; 1-Methylene-2b-Hydroxymethyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C; 2,6,10-Dodecatrien-1-Ol, 3,7, 11-Trimethyl-9-(Phenylsulfonyl)-, (E,E); Lanosterol; 2,2-Dibromocholestanone; 3-O-Acetyl-6-Methoxy-Cycloartenol- and 2-Isopropyl-5-Methylcyclohexyl 3-(1-(4-Chlorophenyl) -3-Oxobutyl)-C, were found to be novel and first time reported from ethanolic root extracts of

Morus indica. Some of the compounds that were detected by the GC-MS analysis were having good biological activities (**Table 13**) that were reported earlier by various researchers.

Table 13: Some compounds with possible biological activity:

Compound name	Biological activity	References
Alpha-amyrin	Anti-inflammatory, anti-diabetic, anti-cancer, anti-arthritic, three times more potent than aspirin	Raman *et al.* 2012.
Octadecanoic acid, ethyl ester	Anti-bacterial and anti-fungal	Elela *et al.* 2009.
N-hexadacanoic acid	Block HIV-1 entry and infection. Anti-inflammatory, antioxidant, hypocholesterolemic nematicide, 5-alpha reductase inhibitor.	Lee *et al.* 2009. Abubakar and Majinda 2016.
Oleic acid	Antibacterial.	Abubakar and Majinda 2016.
Pentadecanoic acid	Lubricant and adhesive agents.	Arora and Kumar 2017.
Urs-12-En-28-Ol	Antimicrobial and anti-inflammatory activity.	Deepa and Selvakumar 2014.
Ethyl-iso-allocholate	Antimicrobial, diuretic, anti-inflammatory, Anti-asthma. Anticancer.	Muthulakshmi *et al.* 2012. Zekeya *et al.* 2014.
9, 12- Octadecadienoic acid (Z,Z)-	Anti-inflammatory, Hepatoprotective, Antiacne, Anticancer, Antiarthritic, Hypocholesterolemic, Nematicide, Anti-coronary, Alpha reductase inhibitor, Insectifuge, Antihistaminic, Antieczemic, Antiandrogenic.	Rajeswari *et al.* 2012.
1-Methylene-2b-hydroxymethyl-3,3 -dimethyl-4b-(3-methylbut -2-enyl)-cyclohexane.	Anti-inflammatory, Anti-hyperlipidemic, Antimicrobial.	Jasmine *et al.* 2013.

Oxidative stress and inflammation are not always harmful, they help phagocytes to kill microorganisms and modulate signaling events through redox regulation (Gordillo *et al.*

2017; Biswas 2016; Kasote *et al*. 2015). However, unregulated and prolonged imbalance in the liver between the production of free radicals and/or reactive oxygen species (ROS) and their elimination by protective mechanisms (antioxidants) leads to damage of important biomolecules and cells, with potential impact on whole organism causing many chronic diseases (Ksouri *et al.* 2015). Various studies have also shown that *M. indica* is having anti-inflammatory activity (Chatterjee *et al.* 1983; Balasubramanian 2005; Oh *et al.* 2010).

Numerous studies have demonstrated the effects of CCl_4 and its interventions on the liver. CCl_4 is a classic compound commonly used for xenobiotic-induced hepatic injury to explain the pathogenesis of hepatic steatosis in the experimental animal model (Johnston & Kroening 1998; Chen *et al.* 2012; Ma *et al.* 2015; Lee *et al.* 2019). The liver injury is due to the ROS-induced oxidative stress that can generate toxic lipid intermediates (Ksouri *et al.* 2015). Weber *et al.* 2003, understood in their study that CCl_4 ingestion activates cytochrome system (i.e., CYP2E1) and forms trichloromethyl radicals (CCl_3). It becomes toxic because of a reactive intermediate generated by its reductive metabolism, and this highly reactive intermediate is known to induce leakage of serum enzymes (AST, ALT, ALP, and GGT), lipid peroxidation, depletion of antioxidant capacity and hepatic necrosis around the central vein. There are other important indices to evaluate the hepatic function, such as TC, TG, HDL, LDL, VHDL, total protein and albumin level in serum.

The current *in-vivo* study demonstrated that the CCl_4-induced control group had a significant increase in the activities of liver indices suggesting acute cellular damage which signifies elevated levels of serum enzymes activities and other indices (Karakus *et al.* 2011; Li *et al.* 2016; Lee *et al.* 2019; Srivastava & Shivanandappa 2010). On the other hand, daily administration of RoMi-EE to CCl_4-induced hepatotoxic rats attenuated the increased activity of liver marker enzymes and alleviated the loss of functional integrity of the cell membrane, indicating its hepatoprotective activity. RoMi-EE at 200 mg/kg concentration was able to pull down the levels of these serum enzymes compared to standard drug silymarin, which showed a better index. Several study, (Wills & Asha 2006; Mani *et al*. 2016) signify that after liver injury, AST and ALT progresses from the cytoplasm to circulatory system because of the toxicity mediated transformed permeability of the cellular membrane.

Creatinine is an important parameter indicating the health of both the liver as well as the kidneys. It was evident from the previous studies that the administration of CCl_4 induced a renal failure indicated by elevation of creatinine level (Abdel Monein & El-Deib 2012; Al-

Yahya *et al.* 2013; Abdulhameed *et al.* 2017). Creatinine is a by-product of muscle metabolism that is excreted unchanged by the kidneys, hence making it an important indicator of renal health. Upon treatment with RoMi-EE, higher concentration (200 mg) showed 0.71 ± 0.04 mg/dL creatinine level which was comparable with that of silymarin (0.70 ± 0.01 mg/dL) with no significant change.

Furthermore, the histopathological examination of the liver provided evidence of the effects of investigated components against acute CCl_4-induced liver injury and also substantiated the biochemical analysis. The histology showed that CCl_4 administration caused serious oxidative liver damage to characterize by severe necrosis, inflammation, hepatocellular degeneration, cytoplasmic vacuolation and loss of cellular boundaries, which confirmed with the previous studies for the live injury (Lee *et al*. 2019; Maheshwari *et al.* 2011; Vuda *et al.* 2012; Nwidu *et al*. 2017). Treatment with RoMi-EE was noteworthy in a dose-dependent manner as it reduced the severity caused due to oxidative damage. This was evidenced by a decrease in necrosis and hemorrhage. Hence, it clearly indicates the protection provided by the administration of RoMi-EE. The high dose of RoMi-EE (**Fig 24**) induced an effect close to normal emergence, recommending that the high dose of 200 mg/kg was more effectual than the lower dose. CCl_4 group wistar rat kidney (cortex) cross section showed vacuolation, glomerular atrophy, widening of capsule space, cell layer thickening and degeneration of cells which were also reported earlier by Abdulhameed *et al.* 2017; Jan & Khan 2016; Yoshioka *et al.* 2016; Sukandar *et al.* 2013. After administration of 100 mg RoMi-EE and 200 mg RoMi-EE experimental drug, the recovery of glomerular atrophy, capsule space decrease and less degeneration of cell was observed. However the 200 mg RoMi-EE experimental drug were better than the low concentration of RoMi-EE and was comparable to that of silymarin having identical structure in kidney histopathology (**Fig 25**) and was also similar to that of normal group.

Antioxidant enzymes, such as SOD, CAT, and GPx are the endogenous enzymes which form an imperative part of the antioxidant defense system. They detoxify the free radicals and thus protect the hepatic cells against oxidant-mediated injury. Treatment with CCl_4 alone can deplete the activity of these enzymes. It also depletes the hepatic GSH system, which is a key component of the overall antioxidant defense system and can also cause lipid peroxidation resulting in liver cirrhosis (Lin *et al.* 2008; Khan *et al.* 2012; Kasote *et al*. 2015). As shown in **Fig 19-21**, the decline in the levels of antioxidant enzymes were observed in CCl_4 treated rats which are clear indicator of excessive formation of hepatic lipid

peroxidation in comparison to the normal group. This has also been reported in previous studies (Tung *et al.* 2009; Maheshwari *et al.* 2011; Lee *et al.* 2019; Ogaly *et al*. 2018; Nwidu *et al*. 2017). On the contrary, the groups treated with RoMi-EE at two different doses significantly increased the levels of SOD, CAT and GPx activities, increased GSH contents and reduced the lipid peroxidation (MDA) level in liver. The preventive effect of RoMi-EE at 200 mg/kg was similar to that of silymarin treatment.

Identification and characterization of binding sites is the key in the process of structure-based drug design (Halgren 2009; Ferreira *et al*. 2015). In some cases there may not be any information about the binding site for a target of interest. In other cases, a putative binding site has been identified by computational or experimental means, but the druggability of the target is not known. Even when a site for a given target is known, it may be desirable to find additional sites whose targeting could produce a desired biological response (Halgren 2009). A new program, called Site Map, is presented for identifying and analyzing binding sites and for predicting target druggability and the Site Score can be used as one criterion for deciding whether to target a given site (Halgren 2009; Patschull *et al.* 2012), but they do not show that a site that scores well is a drug-binding site, sometime it could be (Halgren 2009). Hence, druggability predictions are important to avoid intractable targets and to focus drug discovery efforts on sites offering better prospects (Schmidtke & Barril 2010).

As previously noted, many drug-design projects fail because the target proves not to be druggable (Liu & Altman 2014). Accurate determination at an early stage of whether a given protein is or is not druggable therefore has the potential of saving considerable time and expense (Halgren 2009). Druggability predictions are important to avoid intractable targets and to focus drug discovery efforts on sites offering better prospects and is directly associated to a cavity detection method, screening for druggable cavities in large structural data sets is straight forward (Schmidtke & Barril 2010).

In the current docking study with that of 1NFK protein, the silymarin (CID: 6610285) showed 5 hydrogen bonding with the residues of Gly 162, Glu 179, Asp 220, Ser 221 and Ser 224 of 1NFK site-4, which showed the highest docking score of -5.956. Following the silymarin the compound 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- (CID: 5368759) showed best docking score among the ligands with -4.958 and also showed two hydrogen bonding with residues of Lys 95 and Arg 161. The molecular mechanics/ generalized born suface area (MMGBSA) ΔG binding affinity were in order of

silymarin (-54.79) > 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- (-45.35) > Ethyl Iso-Allocholate- (-39.5) > octadecanoic acid, ethyl ester (-33.85) > 1,2-Bis(Trimethylsilyl) Benzene (-29.05) > Alpha.-amyrin (-27.59) > 2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl- (-26.24) > 1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C (-5.67).

Glushchenko *et al.* 2015, studied flexible molecular docking of chemical compositions of *Bupleurum aureum* plant with NFκB protein (PDB: 1VKX) with "dock into active site" function using Scigress software and found highest docking score in rutin with -3.26. Other notable outcomes are Eicosanoic Acid [CID: 8122]- (Docking score -4.528 & ΔG of -33.85); Ethyl Iso-Allocholate [CID: 6452096]- (Docking score -3.679 & ΔG of -39.5); 1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b-(3-Methylbut-2-Enyl)-C [CID: 550196]- (Docking score -3.277 & ΔG of -5.67); 1,2-Bis(Trimethylsilyl)-benzene [CID: 519794]- (Docking score -3.001 & ΔG of -29.05).

Several studies have also revealed that CCl_4 induction causes increase in concentration of pro-inflammatory markers (*viz*; cytokines, TNF-α, PGE-2, IL-6) due to which elevation in the activity of COX-2 enzymes can be observed which is one of the major inflammatory protein and are also involve in liver injury (Shah *et al*. 2017, Zhang *et al*. 2004, Gunalan *et al.* 2014; Oh *et al.* 2010).

Docking study with that of 3LN1 revealed that the compound: 2,6,10-Dodecatrien-1-Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E), showed best docking score (without violating Lipinski *et al*. 1997, rule of five) among ligands in both docking with 1NFK and 3LN1 proteins. Although the silymarin had shown best docking score in 1NFK receptor, but it didn't show any type of interaction with that of 3LN1 receptor, which clearly indicates the silymarin compound as target specific activity. Amaravani *et al*. 2012, showed that COX-2 has the active binding site of Ala 185, Phe 186, Phe 187, Ala 188, Gln 189, His 190, Thr 192, His 193, Gln 194, Phe 196, Thr 198, Asn 368, Leu 370, Tyr 371, His 372, Trp 373, His 374, Leu 376, Leu 377, Val 433, Ser 437, Gln 440, Tyr 490, Leu 493, Leu 494 amino acids at the active binding pocket. The ligand 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E), have showed Pi-Pi interaction with that of aromatic ring of Trp 373 of the target protein and also showing the best ΔG binding affinity of -27.8173 kcal/mol and was better than the Celecoxib (standard ligand with -17.27 kcal/mol) reported earlier by Lamie *et al.* 2015. The other ligands which showed interaction with both 1NFK and 3LN1

proteins without violating Lipinski rule of five (Lipinski *et al.* 1997) are CID: 550196 (1-Methylene-2b-Hydroxy methyl-3,3-Dimethyl-4b- (3-Methylbut-2-Enyl)-C); CID: 519794 (1,2-Bis(Trimethylsilyl) Benzene) and CID: 610038 (2,4,6-Cycloheptatrien-1-One, 3,5-Bis-Trimethylsilyl-).

The adsorption distribution metabolism and excretion (ADME) properties of ligands revealed that, except for the ligands Octadecanoic acid, ethyl ester (CID: 8122), alpha.-amyrin (CID: 73170), 9,12-Octadecadienoic Acid (Z,Z)-(3931), 1-Octadecyne (69425), Oleic Acid- (445639) and N-Hexadecanoic Acid (985), all the other ligands were in the acceptable range of Lipinski's rule of five (Lipinski *et al.* 1997) and the log P value of the ligands (except CID: 8122, 73170, 3931, 69425 and 445639) also suggests that it cannot cross the blood–brain barrier and hence it can be used as drug in other organs or body parts without affecting the brain tissue (Raghavan *et al.* 2012), indicating their potential for use as drug-like molecules.

Based on the docking study it was revealed that the ligand 2,6,10-Dodecatrien-1- Ol, 3,7,11-Trimethyl-9-(Phenylsulfonyl)-, (E,E)- is comparable with that of silymarin while docking with 1NFK protein and also showed high docking score as well as binding affinity with 3LN1 docking study. From the above outcome it can be proposed that the compound can be utilized for inhibiting NFκB as well as COX-2 inflammatory proteins.